SUMMARY

To carry out reverse genetics experiments with Pelargonium flower break virus (PFBV), one of the most important viruses affecting Pelargonium spp., cDNA clones were constructed from which RNA transcripts can be synthesized in vitro. Two populations of overlapping RT-PCR products encompassing the complete PFBV RNA were ligated into pUC18 with the T7 RNA polymerase promoter fused to the 5' extremity of the viral cDNA. The RNA transcripts derived from one of the resulting clones were infectious when mechanically inoculated to the experimental host Chenopodium quinoa and to the natural host P. zonale inducing local and systemic infections, respectively. The sequence of the infectious cDNA was almost 98% identical to that determined previously for a Spanish isolate of PFBV. This is the first description of a biologically active PFBV cDNA clone, an essential tool for detailed analyses of the viral genome.

Key words: PFBV, infectious transcripts, cDNA clone, Pelargonium.

PFBV is a member of the genus Carmovirus (family Tombusviridae) and, like other carmoviruses, produces icosahedral virions that encapsidate a linear positive-sense single-stranded RNA (Lommel et al., 2005). The complete nucleotide sequence of PFBV genomic RNA has been determined recently (Rico and Hernández, 2004). It comprises 3,923 nucleotides (nt) and contains five open reading frames (ORFs). The 5' proximal ORF encodes a 27 kDa protein (p27) and terminates with an amber codon which may be read-through into an in-frame p56 ORF to generate an 86 kDa protein (p86) that contains viral RNA-dependent RNA polymerase (RdRp) motifs. Two small ORFs, located in the central part of the virus genome, encode polypeptides of 7 (p7) and 12 kDa (p12), respectively, which are probably involved in virus movement. The 3' proximal ORF encodes a 37 kDa capsid protein (CP).

Due to the importance of PFBV in Pelargonium production and because of our interest in the dissection of viral genome elements involved in different steps of the infectious cycle, we generated a full-length viral cDNA clone from which biologically active transcripts could be synthesized in vitro.

Sap from a Pelargonium zonale (Pelargonium x hortorum Bailey) plant collected in Spain and naturally infected by isolate SP18 of PFBV, was mechanically inoculated to Chenopodium quinoa. Total RNA preparations from infected C. quinoa leaves were obtained by phenol extraction and lithium chloride precipitation (Verwoerd et al., 1989) and used as templates for RT-PCR reactions. Different attempts to amplify the complete viral sequence in one step were unsuccessful. To avoid this problem, two different cDNAs were generated with Superscript II-RT (Invitrogen, San Diego, CA, USA): cDNA I, which was synthesized using primer CH44 (5'-CGAGTCGACATTTATGTCCTTCATG-3'), complementary to nt 1,679-1,704 of the PFBV genomic RNA, and cDNA II, which was obtained with primer CH52 (5'-GGTCTAGAGGCGGGTTAAGGTCCTTCATG-3'), complementary to the 3' terminus of the viral sequence (nt 3,903-3,923) with an XbaI site (underlined) at the 5' end. Amplification of cDNA I was performed with the Expand High Fidelity PCR System (Roche) using primers CH44 and CH49 (5'-CGGCATG-3').
CAAGCTTGAATACGACACGACTATAAGGATA-
CATACACTCGGTATCTGG-3'), which contains a
HindIII site (underlined) fused to a T7 RNA polymerase
promoter sequence (in bold) followed by 22 nt of the 5'
end of the virus sequence. PCR amplification of cDNA
II was done using primers CH52 and CH29 (5'-AT-
GAAGGACATAATTGTGCAGTCG-3'), homologous to
nt 1,680-1,699. RT-PCR products obtained with
primers CH44 and CH49 were digested with HindIII
and SalI while RT-PCR products obtained with primers
CH52 and CH29 were digested with SalI and XhoI. The
two digested RT-PCR products were ligated at the SalI
site (present in the PFBV sequence at nt 1,696-1,701 and
embedded in primers CH44 and CH29) and cloned into
HindIII and XhoI sites of pUC18. Recombinant clones
were confirmed as such by restriction analysis. This
approach allowed simultaneous generation of a large pool
of independent clones and was similar to the population
cloning strategy used previously to synthesize full-length
cloned cDNA clones that were sequenced, the in vitro
transcripts should contain five extra nucleotides at the 3' terminus
compared with the wild type viral RNA. No cap analog
was included in the in vitro transcription reactions as the
cap structure is not required for infectivity of viral RNA
of members of the family Tombusviridae (Rochon, 1999).
Batches of four C. quinoa plants were mechanically inocu-
lated (three leaves per plant) with the RNAs derived
from each clone (approximately 0.7 µg per leaf) including
one mock inoculated batch as a negative control. Seven
days after inoculation, only the leaves inoculated with
one of the clones (designated as pSP18-IC) developed
chlorotic lesions that were identical to those produced by
the wild type virus (Fig. 1A). The infectivity of pSP18-IC
was confirmed in numerous independent experiments and
plant infection was further verified by Northern
analysis (Fig. 1B). In addition, viral double-stranded
RNAs were isolated from the infected leaves (Morris and
Dodds, 1979) and polyadenylated using yeast poly(A)
polymerase (U. Biochemical Amersham, Little Chal-
font, England) according to the manufacturer’s instruc-
tions. After phenol-chloroform extraction and ethanol
precipitation, the polyadenylated RNAs were reverse
transcribed using GeneRacer Oligo dT oligonucleotide
(Invitrogen, San Diego, CA, USA), which has a 3' termin-
al 18 nt dT tail and contains the priming sites for the
GeneRacer 3' and GeneRacer 3' Nested oligonucleotides
(Invitrogen, San Diego, CA, USA) at 5' end. RT products
were PCR amplified using either primer GeneRacer 3’ or
GeneRacer 3’ Nested in combination with CH11 (5’-
CATTACACTCGGTATCTGG-3’), homologous to nt
3,704-3,723, to amplify the 3’ terminal region. Sequenc-
ing of the PCR products showed the progeny viral
RNA had the precise 3' terminus indicating that the ex-
tra nucleotides of the original transcripts are removed
during replication in plants.

The full-length cDNA inserted in pSP18-IC was
completely sequenced (Accession number: DQ256073).
Comparison of its nucleotide sequence with that report-
ed previously (Rico and Hernández, 2004) for another
Spanish PFBV isolate, showed an overall sequence iden-
tity of 97.7%. Only ten of the 94 nucleotide substitutions
detected resulted in amino acid changes, five af-
ected the RdRp (one of them non-conservative, Ser to
Phe at position 511 of p86), one affected p7 (non-con-
servative, change Ser to Pro at position 19), two affected
p12 (both of them non-conservative, changes Thr to Ile
at position 12 and Val to Gly at position 14) and two af-
tected the CP.

To get insights into the lack of infectivity of the other
clones that were screened, the nucleotide sequence of
one of them was also fully determined. One nucleotide
insertion was detected at position 2,371 that led to a frameshift within the p7 and p12 genes. This mutation
may have been introduced during reverse transcription and/or PCR because it seems unlikely that non-viable
PFBV variant was present in the initial viral RNA. This
observation supports the rationale of using a population
cloning strategy.

Fig. 1. A) Symptoms induced by in vitro transcripts derived from pSP18-IC in C. quinoa leaves. The picture was taken seven days post-inoculation. B) Northern blot hybridization of total RNA preparations from mock inoculated leaves (U) and from leaves inoculated with in vitro transcripts derived from pSP18-IC (IC). Two micrograms of total RNA samples were denatured by glyoxal-dimethyl sulfoxide treatment, electrophoresed in 1% agarose gels, blotted to nylon membranes, and hybridized with a 32P-labeled DNA probe derived from the 3’ terminus of the PFBV genome. The arrowhead points to the genomic viral RNA; lower bands correspond to subgen-
omic RNAs.
In vitro transcripts derived from pSP18-IC were also inoculated to *P. zonale*, a natural host of PFBV. Except for one case in which chlorotic spots were observed on one leaf, the inoculated plants did not develop obvious symptoms under greenhouse conditions but the virus was detectable by dot-blot hybridization in both inoculated and systemic leaves (Fig. 2A). The infection was further confirmed by RT-PCR analysis with primers CH3 (5’-CGATATCTCAAGAAATTCGAAC-3’), homologous to nt 1,281-1,302 of the PFBV genomic RNA, and CH4 (5’-TCATGAGGTGCCTCGTTATG-3’), complementary to nt 1,664-1,683, which yielded a DNA of the expected size (~400 nt) from extracts of pSP18-IC inoculated plants but not from healthy controls (Fig. 2B). The usual lack of symptoms in the pSP18-IC infected plants parallels the behaviour of the original isolate, which only induced symptoms under certain, not well defined, conditions. This is in agreement with the results of a recent survey showing that the vast majority of naturally PFBV-infected plants are symptomless but some of them may develop chlorotic mottle or petal colour breaking at high temperatures or under water stress (Alonso and Borja, 2005), thus illustrating the strong influence of environmental parameters on symptom elicitation.

To conclude, we have produced a full-length clone of PFBV from which infectious transcripts can be obtained. The availability of this biologically active PFBV cDNA clone is currently allowing us to perform a genetic analysis of the virus.

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REFERENCES


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